

# Protease-activated Receptor 2 (PAR<sub>2</sub>) Protein and Transient Receptor Potential Vanilloid 4 (TRPV4) Protein Coupling Is Required for Sustained Inflammatory Signaling\*

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**Background:** Receptors activate channels of sensory nerves to cause inflammation and pain by unknown mechanisms.

**Results:** Protease-activated receptor 2 (PAR<sub>2</sub>) stimulated transient receptor potential vanilloid 4 (TRPV4) by generation of channel agonists. This required a key TRPV4 tyrosine and induced inflammation.

**Conclusion:** PAR<sub>2</sub> opens TRPV4 by functional coupling.

**Significance:** Antagonism of PAR<sub>2</sub>-TRPV4 coupling could alleviate inflammation and pain.

G protein-coupled receptors of nociceptive neurons can sensitize transient receptor potential (TRP) ion channels, which amplify neurogenic inflammation and pain. Protease-activated receptor 2 (PAR<sub>2</sub>), a receptor for inflammatory proteases, is a major mediator of neurogenic inflammation and pain. We investigated the signaling mechanisms by which PAR<sub>2</sub> regulates TRPV4 and determined the importance of tyrosine phosphorylation in this process. Human TRPV4 was expressed in HEK293 cells under control of a tetracycline-inducible promoter, allowing controlled and graded channel expression. In cells lacking TRPV4, the PAR<sub>2</sub> agonist stimulated a transient increase in [Ca<sup>2+</sup>]<sub>i</sub>. TRPV4 expression led to a markedly sustained increase in [Ca<sup>2+</sup>]<sub>i</sub>. Removal of extracellular Ca<sup>2+</sup> and treatment with the TRPV4 antagonists Ruthenium Red or HC067047 prevented the sustained response. Inhibitors of phospholipase A<sub>2</sub> and cytochrome P450 epoxygenase attenuated the sustained response, suggesting that PAR<sub>2</sub> generates arachidonic acid-derived lipid mediators, such as 5',6'-EET, that activate TRPV4. Src inhibitor 1 suppressed PAR<sub>2</sub>-induced activation of TRPV4, indicating the importance of tyrosine phosphorylation. The TRPV4 tyrosine mutants Y110F, Y805F, and Y110F/Y805F were expressed normally at the cell surface. However, PAR<sub>2</sub> was unable to activate TRPV4 with the Y110F mutation. TRPV4 antagonism suppressed PAR<sub>2</sub> signaling to primary nociceptive neurons, and TRPV4 deletion attenuated PAR<sub>2</sub>-stimulated neurogenic inflammation. Thus, PAR<sub>2</sub> activation generates a signal that induces sustained activation of TRPV4, which requires a key

tyrosine residue (TRPV4-Tyr-110). This mechanism partly mediates the proinflammatory actions of PAR<sub>2</sub>.

Injury and inflammation trigger the activation of proteases from the circulation, immune cells, and epithelial tissues that regulate cells by cleaving protease-activated receptors (PARs)<sup>3</sup>, members of a family of four G protein-coupled receptors (GPCRs) (1, 2). By cleaving PARs at specific sites within the extracellular N-terminal domains, proteases reveal tethered ligands that bind to and activate the cleaved receptors. Synthetic peptides that mimic the tethered ligand domains (PAR-activating peptides, APs) can directly activate PARs and are useful tools to probe receptor functions. Once activated, PARs regulate multiple pathophysiological processes, including inflammation, pain, hemostasis, and healing.

PAR<sub>2</sub> is coexpressed with substance P and calcitonin gene-related peptide by a subpopulation of primary spinal-afferent neurons that control neurogenic inflammation and pain transmission (3, 4). Activation of PAR<sub>2</sub> on sensory nerve endings evokes the local release of these neuropeptides, which stimulate extravasation of plasma proteins, infiltration of neutrophils, and vasodilation (neurogenic inflammation). PAR<sub>2</sub> activation also promotes the central release of neuropeptides that activate second-order spinal neurons that transmit pain. These mechanisms contribute to painful inflammation of the intestine (5, 6), pancreas (7, 8), and joints (9). Therefore, it is of considerable

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<sup>3</sup> The abbreviations used are: PAR, protease-activated receptor; GPCR, G protein-coupled receptor; AP, activating peptide; TRPA1, transient receptor potential ankyrin 1; TRPV, transient receptor potential vanilloid; DRG, dorsal root ganglia; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; 4α-PDD, 4α-phorbol didecanoate; BIM-1, bisindolylmaleimide I; EET, epoxyeicosatrienoic acid; 17-ODYA, 17-octadecynoic acid; MAFF, methyl arachidonyl fluorophosphate; PKC, protein kinase C; HBSS, Hanks' balanced salt solution; MPO, myeloperoxidase.

interest to understand the mechanisms by which PARs regulate the activity of nociceptive neurons.

Members of the transient receptor potential (TRP) family of ion channels, including TRP vanilloids 1 and 4 (TRPV1 and TRPV4) and TRP ankyrin 1 (TRPA1), mediate neurogenic inflammation and pain (10, 11) and are major downstream targets of PAR<sub>2</sub> (12–19). Activation of these non-selective cation channels stimulates the influx of extracellular Ca<sup>2+</sup> ions and the release of neuropeptides in peripheral tissues and the spinal cord, which induces neurogenic inflammation and pain. During injury and inflammation, several factors are generated that can directly activate these channels (10, 11). Elevated temperatures, protons, and lipid mediators activate TRPV1 (20–22); mechanical shear stress, osmotic stimuli, and lipid mediators activate TRPV4 (23–25); and products of reactive oxygen species and reactive prostaglandin metabolites activate TRPA1 (26, 27). However, indirect mechanisms, particularly those triggered by GPCRs, play a prominent role in TRP channel activation. Many GPCRs that induce neurogenic inflammation and pain indirectly regulate TRP channels, which mediate their proinflammatory and pronociceptive actions. Sensitization, whereby pretreatment with a GPCR agonist amplifies responses to a TRP channel agonist, is a well recognized mechanism of indirect regulation. For example, agonists of PAR<sub>2</sub> (12–19), bradykinin-B<sub>2</sub> (22, 28), histamine H<sub>2</sub> (16, 29), neurokinin NK<sub>1</sub> (30) and NK<sub>2</sub> (31), prostaglandin E<sub>2</sub> (32, 33), prokineticin PKR1 and PKR2 (34), and purinergic P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors (35, 36) sensitize TRP channels. The mechanisms by which GPCRs sensitize TRPs are not fully understood. However, in a manner that is reminiscent of the process by which rhodopsin sensitizes ancestral TRP channels in the *Drosophila* eye (37), a major mechanism depends on phospholipase C-mediated cleavage of phosphatidyl inositol 4,5 biphosphate in the plasma membrane (17, 38, 39). This mechanism relieves tonic TRP inhibition and activates protein kinases A and C, which phosphorylate TRPs and modify channel gating (12, 22, 40, 41).

In addition to sensitization, emerging evidence suggests that GPCR signaling can directly activate TRP channels. Responses of dorsal root ganglion (DRG) neurons to bradykinin and histamine are largely dependent on Ca<sup>2+</sup> influx through TRPV1 (28, 42). Products of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and lipoxygenase can directly activate TRPV1, including *N*-arachidonoyl ethanolamide, which is derived from membrane phospholipids, and hydroperoxy or hydroxy eicosatetraenoic acids and leukotriene B<sub>4</sub> products of the lipoxygenase-dependent metabolism of arachidonic acid (43). Diacylglycerol directly activates TRPV1 through a kinase-independent mechanism that underlies cellular responses to M<sub>3</sub> muscarinic and glutamate mGluR5 agonists (44, 45). 5-Hydroxytryptamine and acetylcholine activate TRPV4 by a mechanism that is dependent on generation of epoxyeicosatrienoic acid (5',6'-EET and 8',9'-EET) products of cytochrome P450 epoxygenase-dependent catalysis of arachidonic acid (46–48).

We have identified a new mechanism by which PAR<sub>2</sub> activates TRPV4 channels. Although TRPV1, TRPV4, and TRPA1 mediate the pronociceptive actions of PAR<sub>2</sub> (12–19), the mechanisms underlying the functional interactions between TRPs and PAR<sub>2</sub> are not fully understood. HEK293 cells endogenously

express PAR<sub>2</sub> (49) and respond robustly to PAR<sub>2</sub> stimuli, including trypsin or the tethered ligand mimetic peptide, SLIGRL-NH<sub>2</sub>, with a rapid, transient increase in intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>). By modulating the expression of TRP channels, we report the novel finding that TRPV4, but not TRPA1, contributes to the cellular response to PAR<sub>2</sub> agonists in HEK cells. This mechanism involves PLA<sub>2</sub>- and cytochrome P450 epoxygenase-dependent catalysis of arachidonic acid and requires the phosphorylation of a key TRPV4 tyrosine residue (Tyr-110) that has been implicated in sensitization of TRPV4 (50). We propose that the activation of TRPV4 serves to prolong PAR<sub>2</sub> signaling and to amplify its proinflammatory and pronociceptive actions. These findings have implications for the consequences of protease-dependent PAR<sub>2</sub> activation during injury and inflammation.

## EXPERIMENTAL PROCEDURES

**Reagents**—The following reagents were used: mouse/rat PAR<sub>2</sub>-AP (SLIGRL-NH<sub>2</sub>) (CPC Scientific, San Jose, CA); 4 $\alpha$ -phorbol didecanoate (4 $\alpha$ -PDD) and bisindolylmaleimide I (BIM-1) (Calbiochem, La Jolla, CA); 17-octadecynoic acid (17-ODYA) and methyl arachidonyl fluorophosphonate (MAFP) (Enzo Life Sciences, Waterloo, Australia); HC067047 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and capsaicin, GSK1016790A, indomethacin, Ruthenium Red, trypsin, Src inhibitor 1, and ATP (Sigma-Aldrich, St. Louis, MO).

**Site-directed Mutagenesis**—Tyrosines 110, 805, and 110/805 of human TRPV4 were mutated to phenylalanine using the QuikChange<sup>TM</sup> site-directed mutagenesis kit (Stratagene, Santa Clara, CA) according to the directions of the manufacturer. The following primer sequences were used: hTRPV4 Y110F, ATGGACTCACTGTTTGACTTTGGCACCTATCGTCACTCACT (forward) and AGTGGTGACGATAGGTGCCAAAGTCAAACAGTGAGTCCAT (reverse); hTRPV4 Y805F, GGGCAAGAATGAGACCTACCAGTATTATGGCTTC (forward) and GAAGCCATAATACTGGTAGGTCTCATTCTTGCCC (reverse). Sequences were confirmed by automated DNA sequencing.

**Cell Lines**—HEK293 cell lines stably expressing human TRPV4 or rat TRPA1 were generated using a tetracycline-inducible system as described (18, 26, 51). Briefly, Flp-In<sup>TM</sup> T-Rex<sup>TM</sup> HEK293 cells were transfected with pcDNA5/FRT/TO containing human TRPV4 (HEK-TRPV4 cells, TRPV4 untagged or with C-terminal intracellular HA.11 epitope) or rat TRPA1 (HEK-TRPA1 cells) using Lipofectamine 2000 (Invitrogen). Cells were grown in DMEM containing 10% tetracycline-free FBS, hygromycin (50  $\mu$ g/ml), and blasticidin (5  $\mu$ g/ml). To induce TRP channel expression, tetracycline (0.001–0.1  $\mu$ g/ml) was added to the medium 16 h before use. For controls, HEK cells were transfected with an empty vector control, or non-transfected cells were used (HEK control).

**Animals**—Rats (Sprague-Dawley, 200–250 g, male) and mice (*trpv4*<sup>−/−</sup> and *trpv4*<sup>+/+</sup> littermates, 20–30 g, male) (52) were studied. Procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco or the University of Melbourne Animal Experimentation Ethics Committee.

**Immunofluorescence**—HEK-TRPV4-HA cells ( $2.5 \times 10^5$ /35-mm dish) were plated onto poly-D-lysine-coated coverslips (100  $\mu\text{g}/\text{ml}$ ). Cells were incubated with graded concentrations of tetracycline (0–0.1  $\mu\text{g}/\text{ml}$ , 16 h). Cells were washed with PBS (pH 7.4) and fixed (4% paraformaldehyde, 100 mM PBS (pH 7.4), 20 min on ice). Cells were incubated in blocking buffer (1% normal horse serum in PBS with 0.1% saponin,  $3 \times 10$  min) and then incubated with rat anti-hemagglutinin (1:1,000, overnight, 4 °C; clone 3F10, Roche). Cells were washed and incubated with donkey anti-rat IgG coupled to Rhodamine Red-X (1:200, 2 h, room temperature; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Slides were examined using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss Inc., Thornwood, NY). Images were acquired using Zeiss Neofluar  $\times 40$  (numerical aperture 1.3) or Fluar Plan Achromat  $\times 63$  (numerical aperture 1.4) objectives.

**Measurement of  $[\text{Ca}^{2+}]_i$  in HEK Cells**—For population studies, HEK cells were seeded onto 96-well plates (25,000 cells/well) coated with poly-D-lysine (100  $\mu\text{g}/\text{ml}$ ) and cultured for 48 h. Cells were loaded with Fura2-AM ester (2.5  $\mu\text{M}$ , 30 min, 37 °C) in Hanks' balanced salt solution (HBSS) containing 20 mM HEPES and 0.1% BSA. Fluorescence was measured (4-s intervals) at 340 nm and 380 nm excitation and 510 nm emission wavelengths using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA) as described (53). In some experiments, cells were incubated in  $\text{Ca}^{2+}$ -free HBSS containing EDTA (20 mM). Results were expressed as the 340/380-nm fluorescence emission ratio, which is proportional to the  $[\text{Ca}^{2+}]_i$ . For measurement of  $[\text{Ca}^{2+}]_i$  in individual cells, HEK cells were studied at 37 °C using a Leica DMI-6000B imaging system (Leica, Germany) with  $\times 10$  or  $\times 20$  objectives. Images were collected (5 s intervals) at 340 nm and 380 nm excitation and 510 nm emission wavelengths. Images were processed using ImageJ software with McMaster Biophotonics Facility plug-ins (v1.46b) as described (53). Cells were challenged with PAR<sub>2</sub>-AP (50  $\mu\text{M}$ ) or trypsin (300 units/ml). In some experiments, cells were preincubated for 30 min with MAFP (1  $\mu\text{M}$ , 10  $\mu\text{M}$ , PLA<sub>2</sub> inhibitor), 17-ODYA (50  $\mu\text{M}$ , cyclooxygenase inhibitor), indomethacin (50  $\mu\text{M}$ , cyclooxygenase inhibitor), BIM-1 (100 nM, PKC inhibitor), or Src inhibitor 1 (Src1, 10  $\mu\text{M}$ , Src family kinase inhibitor) 30 min before stimulation. Cells were also assayed in  $\text{Ca}^{2+}$ -free HBSS containing EDTA (20 mM) or after treatment with Ruthenium Red (10  $\mu\text{M}$ , TRPV antagonist).

**Measurement of  $[\text{Ca}^{2+}]_i$  in DRG Neurons**—Rats were anesthetized with isoflurane and killed by bilateral thoracotomy. DRG were collected from all spinal levels and cultured as described (13, 53). Neurons were plated onto glass coverslips coated with poly-L-lysine and laminin (100  $\mu\text{g}/\text{ml}$ ) and cultured for 48–72 h. Neurons were loaded with Fura2-AM ester (2  $\mu\text{M}$ , 30 min, 37 °C) in HBSS, washed, and incubated in HBSS for 30 min before study. Responses of individual neurons to agonists were measured by microscopy as described for HEK cells. Neurons were challenged sequentially with trypsin (300 units/ml), GSK1016790A (10 nM, TRPV4 agonist), capsaicin (100 nM, TRPV1 agonist), and KCl (50 mM). In some experiments cells were pretreated with HC067047 (10  $\mu\text{M}$ , TRPV4 antagonist) 30

min prior to addition of agonists or were assayed in  $\text{Ca}^{2+}$ -free HBSS.

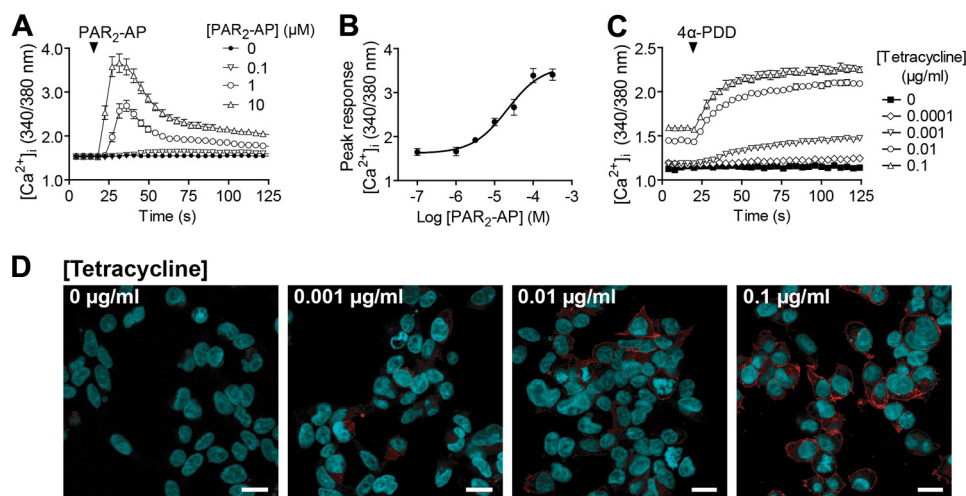
**Analysis of  $\text{Ca}^{2+}$  Signals**—The transient  $\text{Ca}^{2+}$  response induced by PAR<sub>2</sub>-AP challenge of HEK cells was calculated as the difference between the basal 340/380-nm fluorescence emission ratio (average of four readings immediately prior to application of PAR<sub>2</sub>-AP) and the maximal fluorescence that was measured 10–20 s after PAR<sub>2</sub>-AP application. The sustained  $\text{Ca}^{2+}$  response was calculated as the difference between the basal fluorescence and the fluorescence measured at 50–60 s after PAR<sub>2</sub>-AP application. The results were expressed as a ratio of the transient to the sustained response. A diagram illustrating these measurements is presented in Fig. 2B. Representative traces were obtained by averaging values recorded from three different wells with paired vehicle and treated groups. At least three technical repeats were performed for each experiment.

For analysis of DRG,  $\text{Ca}^{2+}$  responses were included of cells that responded to  $\text{K}^+$  stimulation with an increase in 340/380-nm fluorescence emission ratio of  $> 0.1$  units above the initial base line. The trypsin-responsive population was subdivided further on the basis of the magnitude of responses to GSK1016790A, with lower and upper quartiles designated as “low” and “high” responders, respectively. At least 640 neurons were analyzed ( $n = 5$  independent cultures) per treatment group.

**Cell Surface Biotinylation**—Cell surface labeling was performed with EZLink sulfo-NHS-LC-biotin (Pierce) as described in detail (54).

**Western Blotting**—Proteins were resolved in Criterion 4–15% Tris-glycine gels (Bio-Rad), electroblotted onto nitrocellulose membrane (Protran, Whatman, Rydalmere, Australia), and blocked in 5% skim milk/Tris-buffered saline + 0.05% Tween 20 (TBS-T). Membranes were probed with rabbit anti-TRPV4 antibody (1:1,000 in 5% skim milk/TBS-T overnight, 4 °C; Abcam, Waterloo, Australia) and then washed and incubated with IRDye 800 donkey anti-mouse IgG (1:5,000, 1 h, room temperature; Li-Cor Biosciences, Lincoln, NE). Membranes were washed and analyzed using an Odyssey infrared imager (Li-Cor Biosciences). Signal density was quantified using ImageJ software.

**Assessment of Inflammation**—Mice were anesthetized with isoflurane (2%), and baseline paw thickness was measured using a digital caliper (Mitutoyo, Aurora, IL). PAR<sub>2</sub>-AP (50  $\mu\text{g}/\text{paw}$ ) or 0.9% NaCl (50  $\mu\text{l}$ ) were administered by intraplantar injection. The paw thickness was measured from 30–180 min after injection. In some experiments, mice were treated with 17-ODYA (5 mg/kg, 150  $\mu\text{l}$ , intraperitoneal) or vehicle (25% DMSO, 0.9% NaCl, 150  $\mu\text{l}$ , intraperitoneal) 30 min before the intraplantar injections. The paw thickness was normalized to base line (0 min). Mice were killed 6 h after the injection. Paws were collected, snap-frozen in liquid nitrogen, and assessed for tissue myeloperoxidase (MPO) activity as described (55). MPO was solubilized with hexadecyltrimethylammonium bromide, and MPO activity was measured with a dianisidine- $\text{H}_2\text{O}_2$  assay. Changes in absorbance at 450 nm over a 15-min period were determined using a microplate reader (Molecular Devices).



**FIGURE 1. Expression and function of PAR<sub>2</sub> and TRPV4 in HEK cell lines.** A, PAR<sub>2</sub>-AP stimulated a concentration-dependent increase in  $[Ca^{2+}]_i$  in HEK-TRPV4 cells that were not treated with tetracycline. B, the peak increase in  $[Ca^{2+}]_i$  to PAR<sub>2</sub>-AP was concentration-dependent. C, the level of expression of functional TRPV4 was dependent on the tetracycline concentration (0–0.1 μg/ml) used for induction, as demonstrated by elevated  $[Ca^{2+}]_i$  in response to stimulation with 4α-PDD (100 nM). A–C,  $n = 3$  experiments. D, TRPV4-HA expression at the plasma membrane of HEK-TRPV4 cells was dependent on the concentration of tetracycline (0.001–0.1 μg/ml) that was used for induction, as demonstrated by immunofluorescence using HA.11 antibody (red). Nuclei are indicated by DAPI staining (cyan). Scale bar = 5 μm.

Data were expressed as MPO activity relative to total protein (units/mg) and normalized to controls.

**Statistical Analysis**—Results were expressed as the mean  $\pm$  S.E. and were compared by Student's or one-sample *t* test (one-tailed) or one-way analysis of variance and Newman-Keuls test, as indicated, using GraphPad Prism (v5.0). Differences were considered significant when  $p < 0.05$ .

## RESULTS

**PAR<sub>2</sub> Couples to TRPV4, Which Mediates Influx of Extracellular  $Ca^{2+}$  Ions**—We confirmed that Flp-In™ T-Rex™ HEK293 cells express endogenous PAR<sub>2</sub> by examining the effects of graded concentrations of PAR<sub>2</sub>-AP (SLIGRL-NH<sub>2</sub>) on  $[Ca^{2+}]_i$ . PAR<sub>2</sub>-AP caused a transient and concentration-dependent increase in  $[Ca^{2+}]_i$  with a pEC<sub>50</sub> (negative logarithm of the EC<sub>50</sub>) of  $4.67 \pm 0.15$  and a maximal response to 100 μM (Fig. 1, A and B).

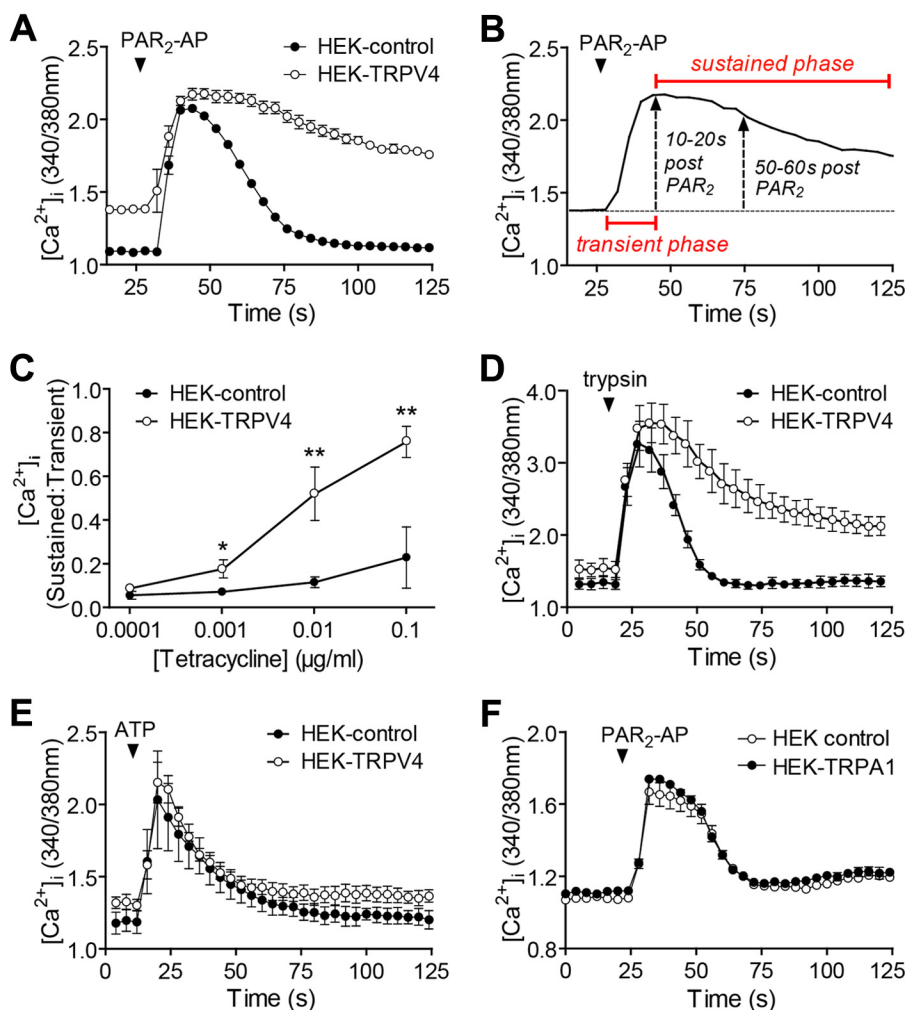
To examine the effect of TRPV4 expression on PAR<sub>2</sub>-evoked  $Ca^{2+}$  signaling, we generated HEK cell lines expressing TRPV4 with or without an HA tag. TRPV4 was expressed under control of a tetracycline-inducible promoter, which enabled controlled expression of the channel. The expression of TRPV4 was examined by measuring changes in  $[Ca^{2+}]_i$  in response to the TRPV4 activator 4α-PDD and by immunofluorescence and confocal microscopy using an antibody to the HA.11 epitope. In HEK-TRPV4 cells not exposed to tetracycline, 4α-PDD had no effect on  $[Ca^{2+}]_i$  (Fig. 1C), and immunoreactive TRPV4 was undetectable (D). Incubation of HEK-TRPV4 cells with graded concentrations of tetracycline (0.0001–0.1 μg/ml, 16 h) induced graded expression of functional and immunoreactive TRPV4. In tetracycline-treated cells, 4α-PDD elicited a gradual increase in  $[Ca^{2+}]_i$  that was sustained for the period of observation (200 s) and was graded with the concentration of tetracycline (Fig. 1C). Responses to 4α-PDD were detected after incubation with 0.001 μg/ml tetracycline and were maximal after 0.1 μg/ml tetracycline. The basal  $[Ca^{2+}]_i$  was also elevated by the highest

expression levels of TRPV4 (0.01, 0.1 μg/ml), although the cells were microscopically normal and remained responsive to TRPV4 and PAR<sub>2</sub> agonists. Immunoreactive TRPV4 was detected in some cells after incubation with 0.001 μg/ml tetracycline and present at the plasma membrane of all cells after incubation with 0.1 μg/ml tetracycline (Fig. 1D).

In HEK control cells (expressing the empty vector without a TRPV4 insert), PAR<sub>2</sub>-AP (50 μM) evoked a transient increase in  $[Ca^{2+}]_i$  that was maximal after 10–20 s and that declined to base line after ~75 s of stimulation (Fig. 2A). In tetracycline-treated (0.1 μg/ml, 16 h) HEK-TRPV4 cells, PAR<sub>2</sub>-AP evoked a similar rapid increase in  $[Ca^{2+}]_i$  that was markedly sustained (Fig. 2A). The sustained response was quantified by determining the ratio of the  $[Ca^{2+}]_i$  at the maximal point of the transient phase (10–20 s) and during the sustained phase (50–60 s) (Fig. 2B). This analysis revealed that the magnitude of the sustained phase was proportional to the concentration of tetracycline and, thus, the level of TRPV4 expression (Fig. 2C). TRPV4 expression similarly enhanced the sustained increase in  $[Ca^{2+}]_i$  to trypsin (300 units/ml), a physiologically relevant PAR<sub>2</sub> agonist (Fig. 2D).

To determine whether TRPV4 similarly affects signaling by other GPCRs, we examined the consequences of TRPV4 expression on responses to ATP, which mobilizes  $Ca^{2+}$  in HEK293 cells by activating P2Y receptors (56). ATP (1 μM) evoked a rapid and transient increase in  $[Ca^{2+}]_i$  in HEK control cells (Fig. 2E). The magnitude and duration of the ATP-evoked  $Ca^{2+}$  response were unaffected by TRPV4 expression ( $p = 0.6$  and  $p = 0.7$  to control cells, respectively). Thus, not all GPCRs can activate TRPV4.

To determine whether PAR<sub>2</sub> can activate other TRP channels, which would represent a more general mechanism of TRP regulation, we examined the effect of expression of TRPA1 on the responses to PAR<sub>2</sub>-AP. TRPA1 was selected because it is coexpressed with PAR<sub>2</sub> by nociceptive neurons and because



**FIGURE 2. TRPV4 expression amplifies the sustained phase of PAR<sub>2</sub> Ca<sup>2+</sup> signaling.** *A*, induction of TRPV4 (HEK-TRPV4, 0.1 μg/ml tetracycline) increased the magnitude and duration of the sustained phase of the Ca<sup>2+</sup> response to PAR<sub>2</sub>-AP relative to non-transfected HEK control cells. *B*, diagram delineating the transient and sustained components of the Ca<sup>2+</sup> response to PAR<sub>2</sub> activation. *C*, quantitative analysis demonstrated that the magnitude of the sustained phase (50–60 s post-PAR<sub>2</sub>-AP) relative to the transient phase (10–20 s post-PAR<sub>2</sub>-AP) (sustained:transient ratio) of the response was dependent on the tetracycline concentration used for induction (0.001–0.1 μg/ml) and, thus, the level of TRPV4 expression. Tetracycline had no effect on control cells. \*, *p* < 0.05; \*\*, *p* < 0.01 compared with HEK control cells for each tetracycline condition (Student's *t* test). *n* ≥ 3 experiments. *D*, trypsin induced a sustained phase only in tetracycline (0.1 μg/ml)-induced HEK-TRPV4 cells. *E*, expression of TRPV4 did not affect the magnitude or duration of the Ca<sup>2+</sup> response to ATP compared with HEK control cells. *F*, TRPA1 expression (HEK-TRPA1, 0.1 μg tetracycline) had no effect on the magnitude or duration of the PAR<sub>2</sub>-AP response compared with HEK control cells.

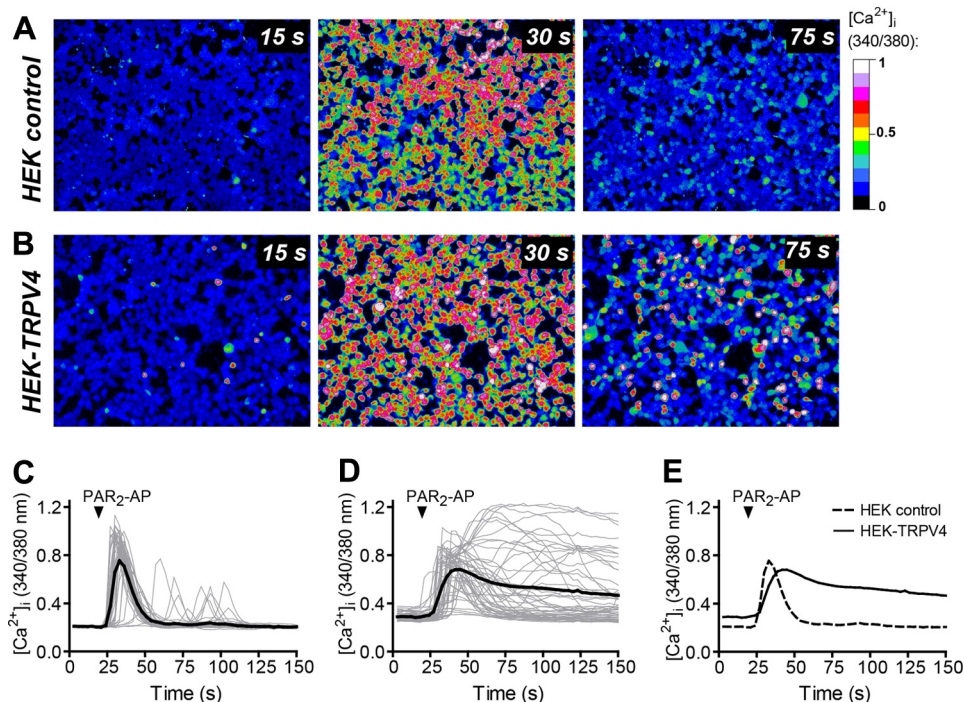
pretreatment with PAR<sub>2</sub> agonists amplifies responses to TRPA1 agonists, which is indicative of channel sensitization. TRPA1 was expressed in HEK cells using a tetracycline-inducible promoter to allow for regulated expression. Tetracycline-induced expression of TRPA1 was confirmed by responsiveness to allyl-isothiocyanate or cinnamaldehyde (data not shown). In contrast to TRPV4, TRPA1 expression did not affect the amplitude or duration of the response to PAR<sub>2</sub>-AP relative to identically treated control cells (Fig. 2*F*). Thus, TRPA1 does not contribute to PAR<sub>2</sub>-evoked Ca<sup>2+</sup> signaling. Addition of the HA epitope tag did not affect PAR<sub>2</sub> or TRPV4 responses to activating stimuli (data not shown). However, all subsequent experiments used untagged TRPV4.

To assess the cell-to-cell variability of PAR<sub>2</sub>-induced activation of TRPV4, we examined responses of individual HEK-TRPV4 cells using microscopy. In HEK-control cells, PAR<sub>2</sub>-AP caused a transient increase in [Ca<sup>2+</sup>]<sub>i</sub>, with no sustained phase (Fig. 3, *A*, *C*, and *E*). In HEK-TRPV4 cells incubated with tetra-

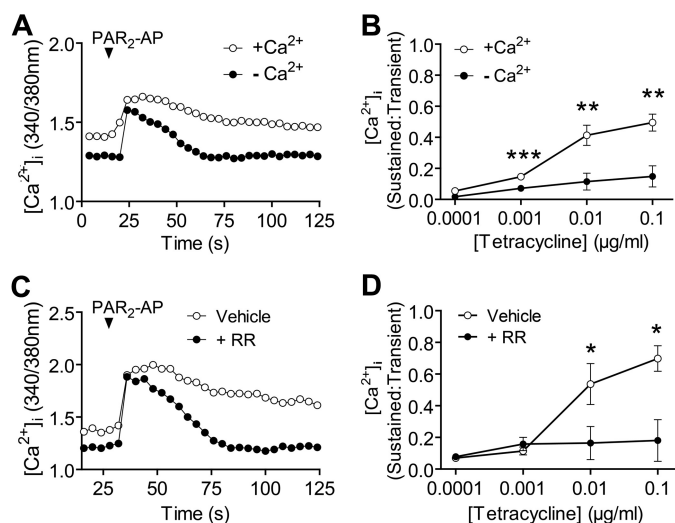
cycline (0.1 μg/ml, 16 h), most cells exhibited a sustained plateau of [Ca<sup>2+</sup>]<sub>i</sub> after treatment with PAR<sub>2</sub>-AP (Fig. 3, *B*, *D*, and *E*). These results indicate that activation of PAR<sub>2</sub> results in a transient increase in [Ca<sup>2+</sup>]<sub>i</sub> and that a more sustained response requires coexpression of TRPV4.

Our results suggest that PAR<sub>2</sub> can activate TRPV4, possibly by generating endogenous TRPV4 agonists or by activating signaling pathways that alter channel gating or localization. We refer to this activation as PAR<sub>2</sub> “coupling” to TRPV4. The mechanism of this coupling is not related to the rapid mobilization of intracellular Ca<sup>2+</sup> *per se* because ATP stimulation of P2Y receptors did not activate TRPV4 despite eliciting a substantial release of intracellular Ca<sup>2+</sup> ions.

**PAR<sub>2</sub> Stimulates an Influx of Extracellular Ca<sup>2+</sup> through TRPV4**—To determine the source of the sustained TRPV4-dependent Ca<sup>2+</sup> response and to further assess the involvement of TRPV4, we either removed extracellular Ca<sup>2+</sup> ions or treated cells with the non-selective TRPV inhibitor Ruthenium Red.



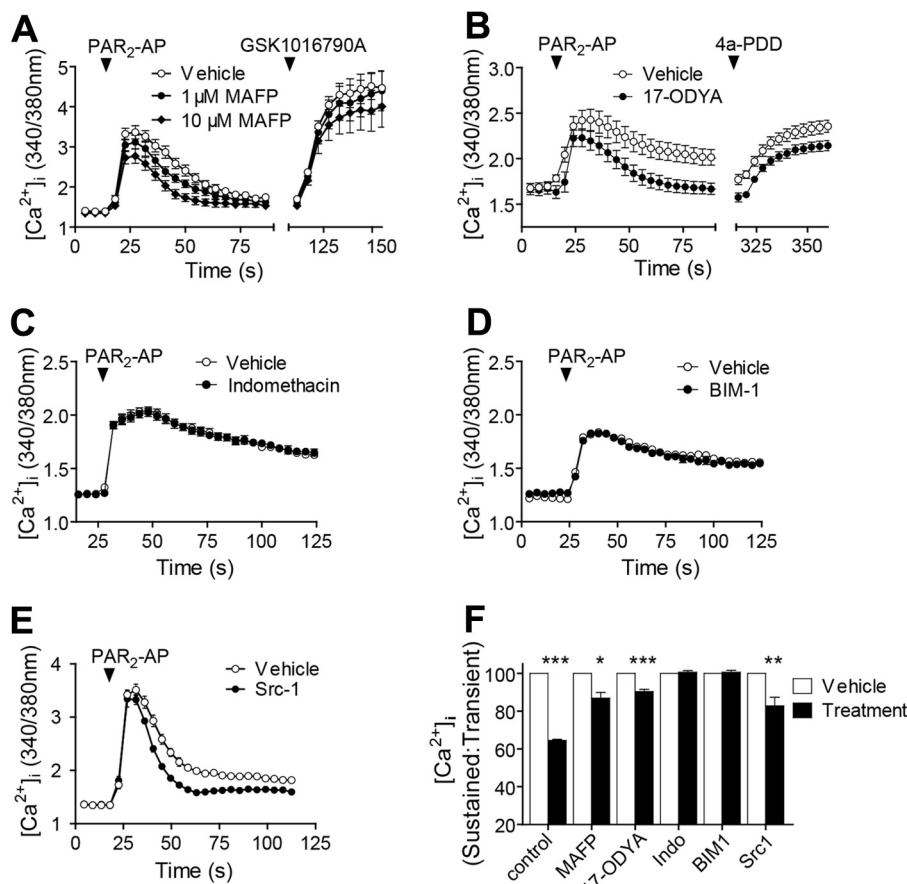
**FIGURE 3. TRPV4 expression amplifies the sustained phase of PAR<sub>2</sub> Ca<sup>2+</sup> signaling in a subset of cells.** A and B, ratiometric images showing non-transfected cells (HEK control, A) and TRPV4-expressing cells (HEK-TRPV4, 0.1  $\mu$ M tetracycline, B) before (15 s) and after (30 s, 75 s) application of PAR<sub>2</sub>-AP (50  $\mu$ M). Images taken at 30 s represent the maximal transient phase of the response to PAR<sub>2</sub>-AP and at 75 s represent the sustained phase. A major subset of cells remains activated at 75 s in the HEK-TRPV4 population. C and D, individual traces from 50 randomly selected cells show responses from HEK-control (C) and HEK-TRPV4 (D) cells when stimulated with PAR<sub>2</sub>-AP. E, mean data showing the augmentation of the sustained phase of the PAR<sub>2</sub> response in TRPV4-expressing cells.



**FIGURE 4. The duration of the PAR<sub>2</sub> [Ca<sup>2+</sup>]<sub>i</sub> response depends on influx of extracellular Ca<sup>2+</sup> ions through TRPV4.** A, in HEK-TRPV4 cells (0.1  $\mu$ M tetracycline), removal of extracellular Ca<sup>2+</sup> attenuated the sustained phase of the Ca<sup>2+</sup> response to PAR<sub>2</sub>-AP compared with the control with extracellular Ca<sup>2+</sup> (representative trace). B, quantitative analysis demonstrated that removal of extracellular Ca<sup>2+</sup> significantly inhibited the sustained phase of the response for all levels of TRPV4 expression. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  compared with the vehicle control for each tetracycline condition (Student's *t* test),  $n \geq 3$  experiments. C, in HEK-TRPV4 cells (0.1  $\mu$ M tetracycline), the non-selective TRPV antagonist Ruthenium Red (RR) attenuated the sustained phase of the Ca<sup>2+</sup> response to PAR<sub>2</sub>-AP compared with the vehicle control (representative trace). D, quantitative analysis demonstrated that Ruthenium Red significantly inhibited the sustained phase of the response for all levels of TRPV4 expression. \*,  $p < 0.05$  compared with vehicle control for each tetracycline condition (Student's *t* test),  $n \geq 3$  experiments.

Omission of extracellular Ca<sup>2+</sup> abolished the sustained phase of the PAR<sub>2</sub>-AP response of tetracycline-induced HEK-TRPV4 cells (Fig. 4, A and B;  $p = 0.002$ ). Ruthenium Red (10  $\mu$ M) also abolished the sustained phase of the PAR<sub>2</sub>-AP response (Fig. 4, C and D). However, removal of extracellular Ca<sup>2+</sup> or addition of Ruthenium Red had no effect on the transient phase of the response to PAR<sub>2</sub>-AP. These results suggest that PAR<sub>2</sub> couples to TRPV4, which mediates an influx of extracellular Ca<sup>2+</sup> ions that comprise the sustained phase of the PAR<sub>2</sub> response.

**PLA<sub>2</sub> and Cytochrome P450 Epoxygenase Contribute to PAR<sub>2</sub>-induced Activation of TRPV4**—PAR<sub>2</sub> couples to PLA<sub>2</sub>, which generates arachidonic acid (57), a substrate for synthesis of endogenous TRPV4 activators including 5',6'-EET (25). We used a pharmacological approach to determine the contribution of PLA<sub>2</sub> and downstream enzymes to PAR<sub>2</sub>-dependent activation of TRPV4. The irreversible PLA<sub>2</sub> inhibitor MAFP (1, 10  $\mu$ M) inhibited both the transient and sustained phases of the response to PAR<sub>2</sub>-AP (Fig. 5, A and F), but lower concentrations of MAFP had no effect (data not shown). Thus, we could not ascribe a selective effect to inhibition of PLA<sub>2</sub> by this compound. MAFP also slightly inhibited TRPV4 activation by the synthetic agonist GSK1016790A (100 nM, Fig. 5A,  $p = 0.045$ ). The cytochrome P450 epoxygenase inhibitor 17-ODYA (50  $\mu$ M) inhibited the sustained phase of the response to PAR<sub>2</sub>-AP without reducing the transient phase of the response (Fig. 5, B and F;  $p = 0.0001$ ). 17-ODYA had a minor inhibitory effect on the response to the TRPV4 agonist 4 $\alpha$ PDD (100 nM), indicating that TRPV4 activity was mostly retained (Fig. 5B). Inhibition of cyclooxy-



**FIGURE 5. PLA<sub>2</sub>, cytochrome P450 epoxygenase, and Src kinase-dependent mechanisms mediate PAR<sub>2</sub> activation of TRPV4.** The effects of inhibitors on PAR<sub>2</sub>-AP-evoked changes in  $[Ca^{2+}]_i$  in HEK-TRPV4 cells (0.1  $\mu$ g/ml tetracycline). **A**, the PLA<sub>2</sub> inhibitor MAFP inhibited the transient and sustained phases of the  $Ca^{2+}$  response to PAR<sub>2</sub>-AP and also had a small inhibitory effect on the response to GSK1016790A. **B**, the cytochrome P450 epoxygenase inhibitor 17-ODYA inhibited the sustained phase of the  $Ca^{2+}$  response to PAR<sub>2</sub>-AP and also had a small inhibitory effect on the response to 4 $\alpha$ -PDD. **C** and **D**, the cyclooxygenase 1/2 inhibitor indomethacin (**C**) and PKC inhibitor BIM-1 (**D**) did not reduce the sustained phase of the  $Ca^{2+}$  response to PAR<sub>2</sub>-AP. **E**, the Src family inhibitor Src 1 did not affect the transient response to PAR<sub>2</sub>-AP but reduced the sustained phase in TRPV4 cells compared with vehicle. **F**, the summarized data demonstrate the effects of inhibitors on the sustained phase by comparing the sustained:transient ratio for inhibitors and matching vehicle treatments. "Control" sustained:transient data compare HEK control and TRPV4-HEK cells (0.1  $\mu$ g/ml tetracycline) from Fig. 2A. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  relative to vehicle; Student's  $t$  test;  $n \geq 3$  experiments.

genase by indomethacin (50  $\mu$ M) did not alter the responses to PAR<sub>2</sub>-AP ( $p = 0.27$ ; Fig. 5, **C** and **F**). Thus, PAR<sub>2</sub> coupling to TRPV4 appears to involve activation of cytochrome P450 epoxygenase, which can generate arachidonic acid-derived TRPV4 activators such as 5',6'-EET.

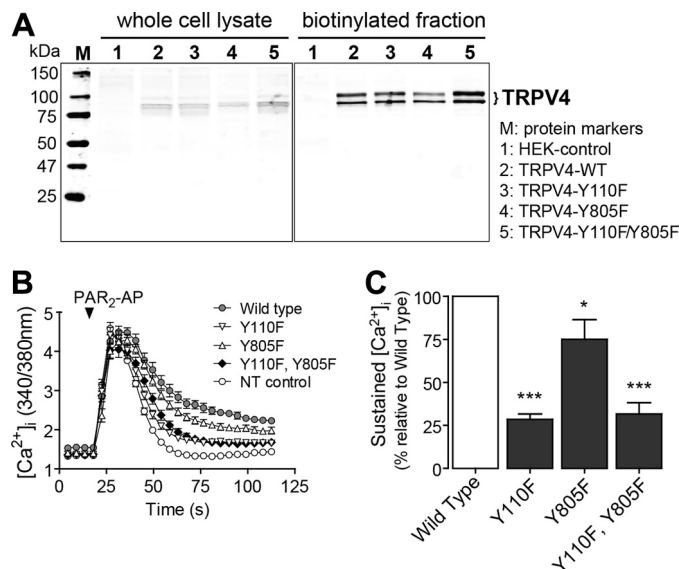
Certain GPCRs, including PAR<sub>2</sub>, can sensitize TRP channels through activation of second messenger kinases, including PKC $\epsilon$ , which can phosphorylate TRPs and regulate channel gating (12, 13, 16–18). Therefore, we examined the effects of kinase inhibitors on PAR<sub>2</sub> coupling to TRPV4. The PKC inhibitor BIM-1 (100 nM) had no effect on the PAR<sub>2</sub>-AP responses (Fig. 5, **D** and **F**). However, the inhibitor of Src-family kinases, Src inhibitor 1 (Src1, 10  $\mu$ M), significantly reduced the sustained phase of the PAR<sub>2</sub>-AP  $[Ca^{2+}]_i$  response without affecting the transient phase of the response (Fig. 5, **E** and **F**;  $p = 0.0028$ ). These data suggest the involvement of tyrosine kinase activity in the TRPV4-dependent sustained phase of the PAR<sub>2</sub> response.

**Tyrosine 110 Is Required for TRPV4 Activation**—To identify the putative sites of tyrosine phosphorylation, we mutated tyrosine residues within the N and C termini of TRPV4 (Y110F,

Y805F, and Y110/805F). Cell surface biotinylation and Western blotting for TRPV4 indicated that wild-type TRPV4 and all TRPV4 mutants were expressed at similar levels at the cell surface (Fig. 6A). PAR<sub>2</sub>-AP induced a rapid and transient increase in  $[Ca^{2+}]_i$  that was similar in cells expressing wild-type and mutant TRPV4 channels (Fig. 6B). However, the sustained phase of the response was markedly attenuated in cells expressing TRPV4-Y110F (Fig. 6, **B** and **C**;  $p < 0.0001$  compared with the wild type). The sustained response to PAR<sub>2</sub>-AP of cells expressing TRPV4-Y805F was only slightly reduced ( $p = 0.03$  compared with the wild type), whereas the response of cells expressing the double mutant TRPV4-Y110/805F resembled that of cells expressing the single mutant TRPV4-Y110F (Fig. 6, **B** and **C**;  $p = 0.001$  compared with the wild type). Responses to the TRPV4 agonist GSK1016790A were unaffected by TRPV4 mutations (data not shown).

These data suggest an involvement of tyrosine kinases in the generation of the sustained phase of the PAR<sub>2</sub> response, presumably through modulation of TRPV4 by phosphorylation of Tyr-110, which we have identified as a key residue required for the TRPV4-dependent response to PAR<sub>2</sub> activation.

**TRP Channels Regulate PAR<sub>2</sub>-dependent Ca<sup>2+</sup> Signaling in DRG Neurons**—To determine whether PAR<sub>2</sub> couples to TRPV4 in cells that naturally express these proteins, we examined

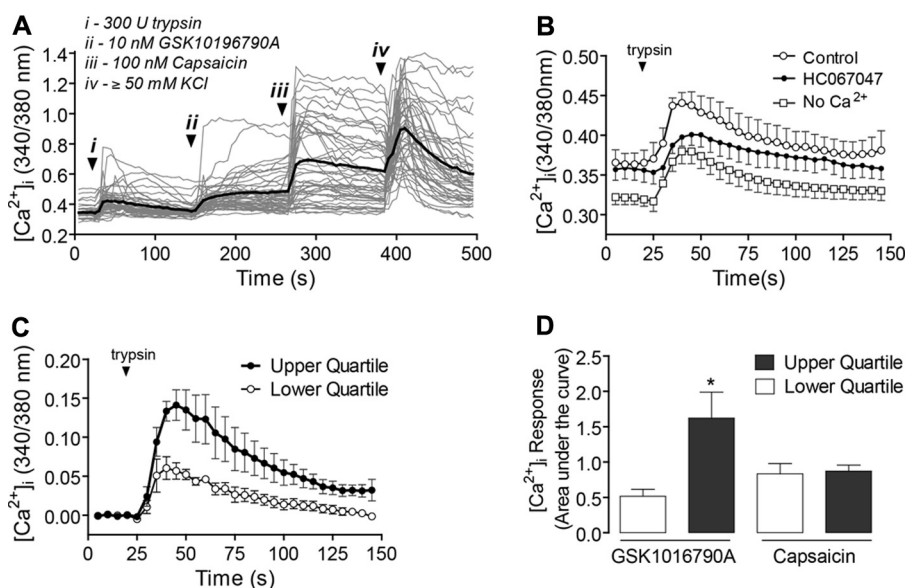


**FIGURE 6. PAR<sub>2</sub>-dependent activation of TRPV4 requires key tyrosine residues.** *A*, TRPV4 WT and the TRPV4 Y110F, Y805F, and Y110F/Y805F mutants were expressed at similar levels at the cell surface of HEK cells and detected by cell surface biotinylation and TRPV4 Western blotting. *B* and *C*, the sustained phase of the Ca<sup>2+</sup> response to PAR<sub>2</sub>-AP was significantly reduced by mutation of the phosphorylation sites Tyr-110 and Tyr-805. A marked reduction in [Ca<sup>2+</sup>]<sub>i</sub> for Y110F and the double mutant (Y110F and Y805F) was evident at 60 s post-PAR<sub>2</sub>-AP treatment ( $p < 0.0001$ ) compared with the Y805F single TRPV4 mutant (expressed as percentage relative to the wild-type control,  $p = 0.03$  (*C*)). \*,  $p < 0.05$ ; \*\*\*,  $p < 0.0001$  relative to the wild type; one sampled *t* test;  $n = 9$  experiments.

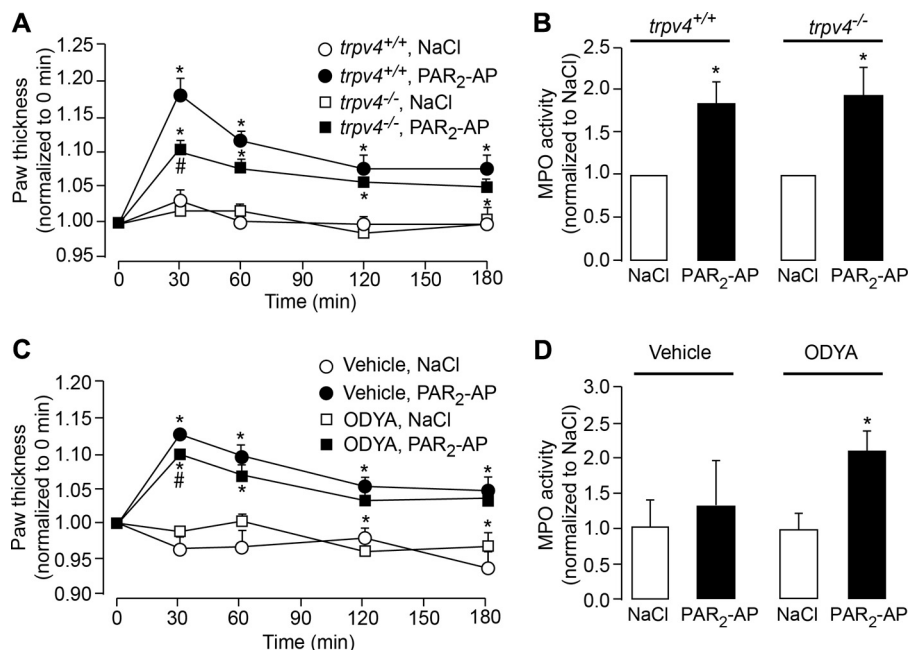
responses of DRG neurons from rats to agonists of PAR<sub>2</sub> and TRP channels. DRG were first challenged with trypsin (300 units/ml), which was selected as a physiological agonist of PAR<sub>2</sub> that gave more robust responses than PAR<sub>2</sub>-AP (data not shown). DRG cultures were then challenged sequentially with the TRPV4 agonist GSK1016790A (10 nM) and the TRPV1 agonist capsaicin (100 nM). Cells were finally exposed to K<sup>+</sup> (50 mM), which depolarizes neurons. Only those cells that responded to K<sup>+</sup> and that were neurons were analyzed. Trypsin stimulated a rapid increase in [Ca<sup>2+</sup>]<sub>i</sub> in neurons, with a peak within 5–15 s that gradually returned to prestimulation levels after 120 s (Fig. 7*A*). Approximately half of all PAR<sub>2</sub>-responsive neurons also responded to GSK1016790A ( $52.78 \pm 12.69\%$ , 649 neurons,  $n = 5$  experiments) with a prompt and sustained elevation in [Ca<sup>2+</sup>]<sub>i</sub>, although the magnitude of those responses was variable. Capsaicin elicited further responses in many of the neurons, which were generally sustained. Not every neuron that responded to GSK1016790A also responded to capsaicin and *vice versa*. These results indicate that PAR<sub>2</sub> and TRPV4 are coexpressed by > 50% of DRG neurons.

The omission of extracellular Ca<sup>2+</sup> ions reduced the basal [Ca<sup>2+</sup>]<sub>i</sub> and blunted the peak and sustained phase of the Ca<sup>2+</sup> response to trypsin (Fig. 7*B*). The TRPV4 antagonist HC067047 (10 μM) did not affect the basal [Ca<sup>2+</sup>]<sub>i</sub> but markedly attenuated the peak and sustained Ca<sup>2+</sup> response to trypsin (Fig. 7*B*).

The variability in responsiveness to GSK1016790A probably reflects variable levels of TRPV4 expression in different neurons. Therefore, we sought to determine whether the magnitude of the PAR<sub>2</sub> responses correlated with the magnitude of the responses to GSK1016790A. To do so, we compared the



**FIGURE 7. PAR<sub>2</sub> activates TRPV4 in DRG neurons.** *A*, Ca<sup>2+</sup> responses of 50 randomly selected rat DRG neurons (gray traces, individual neurons; black trace, mean response of 50 neurons) to sequential stimulation with trypsin (25 s, PAR<sub>2</sub> agonist) (i), GSK1016790A (150 s, TRPV4 agonist) (ii), capsaicin (270 s, TRPV1 agonist) (iii), and KCl (50 mM, 380 s, positive selection for neurons) (iv). *B*, omission of extracellular Ca<sup>2+</sup> ions reduced the baseline [Ca<sup>2+</sup>]<sub>i</sub> and lowered the transient and sustained phases of the response to PAR<sub>2</sub>-AP. The TRPV4 antagonist HC067047 significantly reduced the magnitude of the transient phase ( $0.440 \pm 0.012$  to  $0.391 \pm 0.017$ ;  $p = 0.047$ ) but not the sustained phase ( $0.379 \pm 0.020$  to  $0.357 \pm 0.012$ ;  $p = 0.355$ ) of the PAR<sub>2</sub>-AP response. *N* ≥ 3 experiments. *C* and *D*, the magnitude of the response to GSK1016790A was used to assess TRPV4 expression and activity. Neurons were segregated into upper and lower quartiles of GSK1016790A responsiveness. Analysis of the area under the curve, measured up to 125 s post-PAR<sub>2</sub>-AP, indicated that neurons in the upper quartile exhibited larger transient and sustained responses to PAR<sub>2</sub>-AP compared with neurons in the lower quartile (maximal transient responses at 30 s post-PAR<sub>2</sub>-AP: upper quartile,  $0.137 \pm 0.036$ ; lower quartile,  $0.067 \pm 0.014$ ;  $p = 0.024$ ; Student's *t* test). Neurons were similarly segregated into upper and lower quartiles of capsaicin (100 nM) responsiveness to assess TRPV1 expression and activity (*D*). There was no difference in the PAR<sub>2</sub>-AP response between neurons in the upper and lower quartiles of capsaicin responsiveness ( $p = 0.346$ ). \*,  $p < 0.05$ ; Student's *t* test;  $n = 5$  experiments.



**FIGURE 8. PAR<sub>2</sub>-AP induced paw edema and granulocyte infiltration.** *A*, intraplantar injection of PAR<sub>2</sub>-AP induced sustained paw edema (\*,  $p < 0.05$  compared with NaCl-treated mice;  $n = 8$ –10 mice/group). This effect was significantly attenuated in *trpv4*<sup>-/-</sup> mice compared with *trpv4*<sup>+/+</sup> mice at an early time point (#,  $p = 0.006$  at 30 min;  $n = 10$  mice/group). NaCl injection had no effect. *B*, intraplantar PAR<sub>2</sub>-AP also increased MPO activity in both *trpv4*<sup>+/+</sup> and *trpv4*<sup>-/-</sup> mice ( $p = 0.0028$  and  $p = 0.0088$  compared with NaCl-treated animals,  $n \geq 8$  experiments), but there was no difference between genotypes ( $p = 0.8340$ ). *C* and *D*, pretreatment with 17-ODYA reduced PAR<sub>2</sub>-AP-induced paw edema at 30 min (\*\*,  $p = 0.0305$  compared with PAR<sub>2</sub>-AP/vehicle-treated mice; #,  $p < 0.05$  compared with NaCl-treated mice;  $n = 5$ ) without affecting MPO activity ( $p = 0.019$  compared with NaCl-treated mice;  $n = 5$ ). Analysis of variance and Newman-Keuls test were used.

Ca<sup>2+</sup> response to trypsin, which was quantified as the area under the curve measured up to 125 s after challenge with trypsin, for those neurons in the upper and lower quartiles of the GSK1016790A responses (Fig. 7C). The PAR<sub>2</sub> responses of the neurons in the upper quartile of GSK1016790A responsiveness were ~2-fold greater than the responses of the lower quartile (Fig. 7D,  $p = 0.002$ ). This finding is consistent with a role for TRPV4 in mediating the Ca<sup>2+</sup> responses of the neurons to PAR<sub>2</sub> activation. In contrast, when neuronal populations were similarly subdivided on the basis of their responsiveness to TRPV1 activation with capsaicin, there was no difference between the responsiveness of the upper and lower quartiles (Fig. 7D,  $p = 0.06$ ). Our results indicate that TRPV4 largely mediates the PAR<sub>2</sub>-induced Ca<sup>2+</sup> signals in DRG neurons.

**TRPV4 Mediates PAR<sub>2</sub>-evoked Inflammation**—PAR<sub>2</sub> agonists evoke neurogenic inflammation that depends on the local release of neuropeptides from primary spinal afferent neurons (3, 5). To investigate the functional relevance of PAR<sub>2</sub> coupling to TRPV4 in the intact animal, we assessed the effects of PAR<sub>2</sub>-AP on peripheral inflammation in *trpv4*<sup>+/+</sup> and *trpv4*<sup>-/-</sup> mice. Intraplantar injection of PAR<sub>2</sub>-AP into *trpv4*<sup>+/+</sup> mice resulted in an increase in paw thickness that was maximal within 30 min and maintained for 180 min, indicative of tissue edema and consistent with previous reports (Fig. 8A). The magnitude of PAR<sub>2</sub>-induced paw edema at early time points was reduced in *trpv4*<sup>-/-</sup> mice compared with *trpv4*<sup>+/+</sup> mice ( $p = 0.006$  at 30 min,  $p = 0.052$  at 60 min,  $n = 10$  mice). Paw thickness was similar in both groups at 120 and 180 min after PAR<sub>2</sub>-AP (Fig. 8A,  $p < 0.05$  relative to NaCl-treated mice). No difference in paw thickness was detected between *trpv4*<sup>+/+</sup> and *trpv4*<sup>-/-</sup> mice after intraplantar injection of 0.9% NaCl.

PAR<sub>2</sub>-AP also increased MPO activity in the paws of *trpv4*<sup>+/+</sup> and *trpv4*<sup>-/-</sup> mice (Fig. 8B,  $p = 0.0028$  and  $p = 0.0088$ , respectively, compared with NaCl;  $n = 8$ –10). However, MPO activity was similar in *trpv4*<sup>+/+</sup> and *trpv4*<sup>-/-</sup> mice ( $p = 0.8340$ ).

Pretreatment with 17-ODYA (5 mg/kg, intraperitoneal, 30 min before PAR<sub>2</sub>-AP) reduced the paw edema response to PAR<sub>2</sub>-AP at an early time point (Fig. 8C,  $p = 0.0305$  at 30 min compared with vehicle,  $n = 5$  mice). Paw thickness was similar in both groups at other time points after PAR<sub>2</sub>-AP (Fig. 8C,  $p < 0.05$  relative to NaCl-treated mice). 17-ODYA did not affect the PAR<sub>2</sub>-induced MPO activity (Fig. 8D).

These results indicate that the release of arachidonic acid metabolites and the activation of TRPV4 contributes to the initial phases of PAR<sub>2</sub>-evoked edema. However, TRPV4 is not involved in PAR<sub>2</sub>-mediated recruitment of inflammatory cells.

## DISCUSSION

Our results reveal an unexpected functional coupling between PAR<sub>2</sub> and TRP channels, which involves the generation of an arachidonic acid-derived TRPV4 activator and a tyrosine kinase signaling pathway (Fig. 9). We identified this coupling in a model HEK cell line and in primary nociceptive neurons. The coupling gives rise to long-lasting TRPV4-dependent Ca<sup>2+</sup> signals and demonstrates a direct involvement of TRPV4 in the cellular response to PAR<sub>2</sub> activation, which contributes to the proinflammatory effects of this receptor. We propose that this coupling represents a mechanism of TRP channel regulation that is distinct from the process of sensitization. This convergence of GPCR and TRP signaling may provide a mechanism through which the specificity and magnitude of cellular responses is conferred and controlled. This proposal

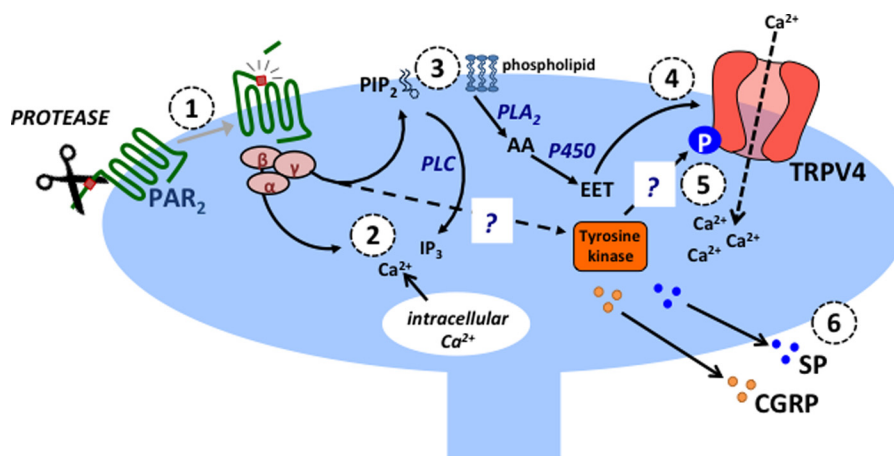


FIGURE 9. **Hypothesized mechanism of PAR<sub>2</sub> and TRPV4-dependent neurogenic inflammation.** Proteases released during inflammation and injury cleave and activate PAR<sub>2</sub> on the terminals of nociceptive spinal afferent neurons (1). PAR<sub>2</sub> activates heterotrimeric G proteins, which leads to release of intracellular Ca<sup>2+</sup> (2) via the phospholipase C (PLC), phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), and inositol 1,4,5-trisphosphate (IP<sub>3</sub>) pathways. PLA<sub>2</sub> hydrolyzes membrane phospholipids to form arachidonic acid (AA), and cytochrome P450 epoxygenase (P450) generates EETs (3). As endogenous TRPV4 agonists, EETs (most likely 5',6'-EET) promote TRPV4 channel opening and influx of extracellular Ca<sup>2+</sup> (4). This requires tyrosine 110, a known target for phosphorylation (P) by tyrosine kinases (5). PAR<sub>2</sub> activation triggers calcium-dependent release of calcitonin gene-related peptide (CGRP) and substance P (SP), which mediate neurogenic inflammation (6). Question marks indicate that the tyrosine kinase remains to be identified and that PAR<sub>2</sub>-induced phosphorylation of TRPV4 has not been examined directly.

is supported by other studies demonstrating functional coupling between GPCRs and TRP channels in native cells (28, 29, 37, 44–48). However, the process of PAR<sub>2</sub> coupling to TRPV4 shows specificity because ATP acting through P2Y receptors did not activate TRPV4, and PAR<sub>2</sub> did not activate TRPA1. These observations suggest that the mechanism of PAR<sub>2</sub> coupling to TRPV4 is not simply due to elevated [Ca<sup>2+</sup>]<sub>i</sub>, which was similarly stimulated by ATP. Two different modes of PAR<sub>2</sub> activation (PAR<sub>2</sub>-AP and trypsin) induced coupling between PAR<sub>2</sub> and TRPV4. Thus, coupling is not an artifact of using the synthetic tethered ligand-based activating peptide and can also occur in response to an endogenous activator.

**Significance of PAR<sub>2</sub>-TRPV4 Coupling for Neurogenic Inflammation and Pain**—PAR<sub>2</sub> agonists promote neurogenic inflammation and pain in multiple tissues (1, 3, 4). TRPV4 is coexpressed with PAR<sub>2</sub> in nociceptive spinal-afferent neurons, and sensitization of these channels following PAR<sub>2</sub> activation is a major mechanism through which both neurogenic pain and inflammation develop (13, 16–18). In our proposed model, the activity of TRPV4 influences the duration of PAR<sub>2</sub>-dependent signaling, thereby augmenting the effects of acute PAR<sub>2</sub> activation. Thus, both coupling and sensitization of TRP channels may contribute to pathophysiological changes associated with PAR<sub>2</sub> activation.

Our experiments with cultured DRG neurons demonstrate that both the magnitude and duration of PAR<sub>2</sub> signaling are modulated by TRPV4 activity, whereas in HEK293 cells only the sustained phase of the PAR<sub>2</sub>-evoked Ca<sup>2+</sup> response was affected by TRPV4 expression. The PAR<sub>2</sub> response was greatly reduced in DRG neurons treated with the TRPV4 antagonist HC067047 or after omission of extracellular Ca<sup>2+</sup> ions. These findings are consistent with preliminary studies showing that Ruthenium Red also suppresses PAR<sub>2</sub> signaling in DRG neurons (data not shown). We conclude that PAR<sub>2</sub>-induced Ca<sup>2+</sup> signaling in DRG neurons depends in large part upon activation of TRPV4 and a TRPV4-mediated influx of extracellular Ca<sup>2+</sup>.

However, a substantial component of the increase in [Ca<sup>2+</sup>]<sub>i</sub> also derives from intracellular Ca<sup>2+</sup> stores.

In this study, we have demonstrated a role for PAR<sub>2</sub>-TRPV4 coupling in the regulation of PAR<sub>2</sub>-mediated paw edema but not in granulocyte infiltration. These findings are consistent with a role for TRPV4 in PAR<sub>2</sub>-dependent signaling in DRG neurons, as the development of paw edema is neurogenic in origin, whereas granulocyte recruitment occurs independently of sensory innervation (3).

**PAR<sub>2</sub>-TRP Channel Coupling in Other Systems**—Our observations may have important functional implications for other cell types in which PAR<sub>2</sub> and TRPV4 are coexpressed, such as bladder urothelium, colonic epithelium, and bronchial epithelial cells (18, 57–60). Whether physiological and pathophysiological responses to PAR<sub>2</sub> activation are augmented by TRPV4 in these tissues has yet to be determined. PAR<sub>2</sub> activation in these cell types leads to increased barrier permeability and is associated with inflammation (61). Similarly, TRPV4 activation results in inflammation and altered cellular permeability of colonic epithelial cells (58). Thus, as with DRG neurons, TRP channels may also regulate PAR<sub>2</sub>-dependent signaling in these cells.

**Signaling Pathways Involved in TRP Activation**—PARs signal through phospholipase C and PLA<sub>2</sub> (57, 62, 63). Arachidonic acid derivatives are endogenous activators of TRPV1, TRPV4, and TRPA1 (24, 25, 43, 64). PAR<sub>2</sub> coupling with TRPV4 involves production of arachidonic acid derivatives, as demonstrated using MAFP, an irreversible inhibitor of PLA<sub>2</sub> (65). This observation is consistent with evidence that PAR<sub>2</sub> activation leads to increased arachidonic acid release and prostaglandin production in enterocytes (57) and with other studies examining GPCR interaction with TRPV4 channels (47). Whether this is a common mechanism in cells that coexpress GPCRs and TRPV4 channels remains to be determined. PLA<sub>2</sub>-derived TRP channel activators contribute to both the transient and sustained phases of the PAR<sub>2</sub> response because MAFP inhibited

both components of the response. The inability of indomethacin to block TRPV4 activation excludes involvement of cyclooxygenase-derived prostaglandin in signaling to TRPV4. Lipoxygenase-derived arachidonic acid derivatives, such as hydroxyeicosatetraenoic acids, are endogenous TRPV1 agonists and are not reported to activate TRPV4 (28, 43, 66, 67). In our experiments, lipoxygenase inhibition reduced cell viability and was not examined further (data not shown). We did not observe coupling between PAR<sub>2</sub> and TRPA1. This result is at variance with a recent study in which 5',6'-EET dependent neuronal activation of TRPA1 was reported (68). The reason for this discrepancy is presently unknown but may relate to the differences in the studied receptors and the experimental systems.

TRPV4 is modulated by a range of serine/threonine and tyrosine kinases and contains target residues, including those for tyrosine kinases (50, 69) and PKC (40). In addition, PKC-activating phorbol esters directly activate TRPV4 (41). We found that PKC-specific inhibition (BIM-1) had no effect on PAR<sub>2</sub> signaling, which excludes a role for PKC in coupling with TRPV4.

Two observations implicate tyrosine phosphorylation of TRPV4 in PAR<sub>2</sub>-TRPV4 coupling. First, the Src family kinase inhibitor, Src1, attenuated TRPV4-dependent PAR<sub>2</sub> signaling. Second, the magnitude of coupling was markedly attenuated by mutation of Tyr-110, a target residue for Src family kinases (50). A limitation of our work is that we have not identified the particular tyrosine kinase involved in PAR<sub>2</sub>-TRPV4 coupling. Although Src1 inhibited coupling, preliminary studies indicated that PP2 was less effective (data not shown). Thus, it is possible that other kinases are involved because nanomolar concentrations of PP2 block certain Src kinases (70). Another caveat of our study is that we did not directly demonstrate phosphorylation of TRPV4-Y110 in response to PAR<sub>2</sub> activation. Further studies using pharmacological and genetic approaches for manipulating kinase activity are required to identify the kinases that mediate tyrosine phosphorylation. Proteomic analyses are necessary to study PAR<sub>2</sub>-induced tyrosine phosphorylation of TRPV4. Once the specific kinase is identified, it will be possible to examine its contribution to protease-evoked inflammation and pain in mice.

**Summary**—In summary, we have demonstrated coupling between PAR<sub>2</sub> and TRPV4. This activation of TRP channels is mediated by production of endogenous activators and is dependent on key tyrosine residues of TRPV4. Our study has identified critical components of the intracellular signaling pathways underlying the activation of TRP channels. These may represent novel targets for therapeutics aimed at reducing augmented signaling under pathophysiological conditions while leaving normal responses intact. This selective inhibition of aberrant signaling is a more attractive target compared with global inhibition of TRP channels, which have important physiological roles in thermoregulation, osmoregulation, and nociception.

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